



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicant : Marie B. CONNETT-PORCEDDU
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COPY

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER RULE 132 OF DAVID S. CANAVERA

Dear Sir:

I, David S. Canavera, declare as follows:

1. My education and experience are as follows. I received a Bachelor of Science degree in Forest Management from Michigan Technological University in 1965 and a Masters of Science degree and a Doctorate degree in Forest Tree Improvement from Michigan State University in 1967 and 1969, respectively. I have been employed by MeadWestvaco Corporation from 1980 to the present. From 1998 to 2002, I have been Director of Forest Research, and I am currently Director of Tree Improvement and Technology at MeadWestvaco. Prior to joining MeadWestvaco, I was Associate Professor of Forest Resources at the University of Maine from 1974 to 1980 and Assistant Professor of Forestry at Tuskegee University from 1972-1974. For several years during my employment at MeadWestvaco, I was also an Adjunct Associate Professor in the Department of Forestry at Clemson University.

2. My experience at MeadWestvaco has involved leading our genetics and biotechnology research programs. In tree improvement, our major research thrusts were developing advanced-generation breeding strategies for loblolly pine and enhancing the genetic quality of seed orchard seed; and development of germplasm for hybrid pines, sweetgum, and cottonwood and other Populus hybrids. In biotechnology, the work focused on pine somatic embryogenesis, conifer

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micropropagation, and pine rooted cuttings; and many areas of molecular biology including genetic transformation of pines and hardwoods, the use of DNA markers in breeding programs, the development of flowering control in trees, stress resistance and development of herbicide resistant trees. Accordingly, I believe that I am well qualified to consider long felt needs in the transformation and regeneration of hard pines.

3. I understand that the claims of the present application recite (a) a method for regenerating transgenic plants of pine of the genus *Pinus* subgenus *Pinus* (claims 1-9 and 11-38), (b) a method for minimizing damage to transformed cell of the pine of the genus *Pinus* subgenus *Pinus* following infection by *Agrobacterium* (claims 39-43 and 45), (c) a method for pine cell tissue culture of pine cells of the genus *Pinus* subgenus *Pinus* (claims 46-51), (d) a method for selecting transformed cells of pine of the genus *Pinus* subgenus *Pinus* (claims 52-57), and (e) a method for eradicating *Agrobacterium* from cells of pine of the genus *Pinus* subgenus *Pinus* (claims 58-62). The method of claims 1-9 and 11-38 provides for enhanced transformation and regeneration of transformed embryogenic pine tissue in which the pine is of the genus *Pinus* subgenus *Pinus*. The remaining methods relate to various aspect of this method. I also understand that the claims of related copending patent application Serial No. 09/973,089 recite a method for regenerating genetically modified plants of pine of the genus *Pinus* selected from the group consisting of Southern yellow pines and hybrids thereof using an improved selection method. This method provides for enhanced regeneration of transgenic embryogenic pine cells for this important group of pines.

4. I understand that the Examiner contends that the invention of the present application, regeneration of transformed embryogenic hard pine (i.e., pines of the subgenus *Pinus*, e.g., *Pinus taeda*), would have been obvious over Levee et al. (*Molecular Breeding* 5:429-440, 1999) which describes the regeneration of transformed embryogenic soft pine (i.e., pines of the subgenus *Strobus*, e.g., *Pinus strobus*). I also have been advised that Levee et al. does not disclose the transformation and regeneration of pine of the subgenus *Pinus*.

5. I have been asked to provide this Declaration concerning a long-felt need in the art for the transformation and regeneration of transformed tissue of pines of the subgenus *Pinus* and the failed attempts of others to meet this need.

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6. The hard pines (i.e., pines of the subgenus *Pinus*), in particular loblolly pine (*P. taeda*, a Southern yellow pine), its elite lines and hybrids, but also including *P. rigida* and *P. radiata*, are the most commercially important pines, and loblolly pine is the most used species of hard pines. Thus, transformation followed by reliable regeneration of the hard pines is more desirable than transformation and regeneration of other conifers, such as white pines (e.g., pines of the subgenus *Strobus*) and spruces. The transformation and regeneration of hard pines has turned out to be more difficult than the transformation and regeneration of other conifers.

7. Researchers have been attempting to transform hard pines with *Agrobacterium* followed by reliable regeneration of the transformed tissue since about 1988-1989. Such research has been conducted in many laboratories, including for example, North Carolina State University, the Institute of Paper Science and Technology, the Canadian Forest Service, International Paper and Westvaco Corporation. During this period, regeneration systems for non-transformed hard pines had been developed. Evidence of the development of regeneration systems for hard pines is illustrated by Handley (U.S. Patent No. 5,491,090; copy attached as Exhibit 1). Although hard pines had been transformed using *Agrobacterium*, regeneration of *Agrobacterium*-transformed hard pines had not been achieved despite considerable effort over this time frame. Evidence that regeneration of *Agrobacterium*-transformed hard pines had not been achieved is illustrated by Wenck et al. (1999, *Plant Mol Biol* 39:407-416; copy attached as Exhibit 2) which specifically states “[w]e have not been able to recover stable transformants through selection to date.” See page 413, bottom of left column with respect to loblolly pine, a hard pine. The experiments of others did not result in regenerated transgenic hard pine, such as loblolly pine, plants as evidenced by Wenck et al. and the dearth of published reports on regenerated transformed loblolly pine plants. Thus, there was a long-felt need to develop improved methods of *Agrobacterium* transformation of hard pines and improved selection of transformed tissue of hard pines, and to develop methods to regenerate *Agrobacterium*-transformed hard pines. This long felt need was not satisfied by transformation of other conifers, such as white pines and spruces.

8. The method for hard pine transformation and regeneration described and claimed in patent application Serial No. 09/973,088 and the method for selection of transgenic Southern yellow pine tissue described and claimed in patent application Serial No. 09/973,089 are the first methods

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which achieved reliable and efficient regeneration of transgenic hard pine plants, i.e., the regeneration of transformed hard pine tissue. This reliable and efficient regeneration of transgenic hard pine results directly from the methods described and claimed in these applications. The methods described and claimed in these patent applications are sufficiently robust to fill the long-held need because they result in the regeneration of transgenic hard pines and they have been shown to be valid for a wide variety of genotypes, including multiple species of hard pines, and even lines from within elite families of loblolly pine such as those being tested by MeadWestvaco Corporation for commercial deployment. Without these methods, reliable and efficient regeneration of transgenic hard pine had not been achieved. Thus, these methods meet the long-felt need of providing regenerated *Agrobacterium*-transformed hard pines.

9. In addition, a method such as the one used by the Canadian Forest Service that is not able to be used for multiple species did not meet the long felt need for regeneration of transgenic hard pines. Thus, the method described and claimed in patent application Serial No. 09/973,088 enabling regeneration of transgenic hard pines does satisfy this long-held need. Furthermore, a method that did not enable selection even from within elite families of loblolly pine would not meet the long felt need. Thus, the method described and claimed in patent application Serial No. 09/973,089 enabling selection even from within elite families of loblolly pine does satisfy this long-held need.

10. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or of any patent issued thereon.

2/13/2004

Date

David S. Canavera

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High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*)

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Abstract

Agrobacterium-mediated gene transfer is the method of choice for many plant biotechnology laboratories; however, large-scale use of this organism in conifer transformation has been limited by difficult propagation of explant material, selection efficiencies and low transformation frequency. We have analyzed co-cultivation conditions and different disarmed strains of *Agrobacterium* to improve transformation. Additional copies of virulence genes were added to three common disarmed strains. These extra virulence genes included either a constitutively active *virG* or extra copies of *virG* and *virB*, both from pTiBo542. In experiments with Norway spruce, we increased transformation efficiencies 1000-fold from initial experiments where little or no transient expression was detected. Over 100 transformed lines expressing the marker gene β -glucuronidase (GUS) were generated from rapidly dividing embryogenic suspension-cultured cells co-cultivated with *Agrobacterium*. GUS activity was used to monitor transient expression and to further test lines selected on kanamycin-containing medium. In loblolly pine, transient expression increased 10-fold utilizing modified *Agrobacterium* strains. *Agrobacterium*-mediated gene transfer is a useful technique for large-scale generation of transgenic Norway spruce and may prove useful for other conifer species.

Introduction

Transformation of conifers has traditionally been a slow and tedious process [reviewed by 10, 36, 37].

Currently, the most successful method is the 'gene gun' or biolistic technique. However, transformation frequencies are still quite low. *Agrobacterium tumefaciens*, which causes crown gall disease in plants by transferring a piece of DNA (T-DNA) from the bacterium to the plant cell, has been shown to transform conifers at low frequency [11, 38, 40]. *Agrobacterium*-mediated gene transfer is the method of choice for most plant biotechnology laboratories because of the high percentage of single copy and single locus insertion events compared to other plant transformation techniques such as biolistics; however, attempts to use *Agrobacterium* for genetic transformation of

conifers followed by subsequent regeneration of whole plants has been largely unsuccessful with the notable exception of European larch (*Larix decidua*; [22]).

Agrobacterium-mediated transformation is influenced by a large number of variables including the strain used, the presence of inducers such as acetosyringone and the type of plant material used for co-cultivation. Plant cells must be actively dividing for transformation [1, 4, 35]. Embryogenic suspension cultures provide a reliable source of rapidly dividing cells. Further, the production and maintenance of these cultures has been reported for many conifer species [42]. We have chosen Norway spruce for these experiments because of the rapidity of cell proliferation, the long-term viability and the regeneration potential of embryogenic cultures [46]. We have also used loblolly

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pine because the potential economic impact of genetically modified pines in the southeastern United States warrants the development of better transformation techniques.

Recently, transformation of previously 'recalcitrant' species (including the economically important species rice and maize) has been accomplished through the use of disarmed *Agrobacterium* helper strains to which additional copies of the virulence regions have been added [14, 18, 19, 23, 25, 26, 29]. Using this strategy, Drake *et al.* [9] were able to show transient expression of a GUS reporter gene in Sitka spruce (*Picea sitchensis*) but no stable transformed lines were produced. We have put additional copies of either a constitutively active *virG* gene from an octopine plasmid (pAD1289; [18]) or a 15.8 kb fragment containing the *virB* and *virG* regions from the 'super-virulent' plasmid pTiBo542 (pToK47; [25]) into either an octopine (LBA 4404; [20]), nopaline (GV 3101 (pMP 90); [27]) or an agropine (EHA 105; [21]) disarmed *Agrobacterium* strain. A binary vector containing a kanamycin resistance gene and an intron containing β -glucuronidase (GUS) gene (pBISN1; [31]) was also introduced into these agrobacteria. Transient expression was observed with embryogenic cultures of both loblolly pine and Norway spruce. In addition, stable transformants were obtained from Norway spruce.

Materials and methods

Plasmids and bacterial strains

The plasmids used in this study were pAD1289 [18], pToK47 [25], pBISN1 [31] and pWWS006 (Wenck, unpublished) and are described in Table 1.

The bacterial strains used in this study were *Escherichia coli* HB101, JM109 or NovaBlue (Novagen, Madison, WI) and *A. tumefaciens* (Table 1) LBA4404 [20], GV 3101 ((pMP 90); [27]) and EHA 105; [21]. *Agrobacterium* was transformed by electroporation as recommended by the manufacturer (Gibco-BRL, Gaithersburg, MD). Selection for transformed cells was as in Table 1 except for LBA4404 which is sensitive to carbenicillin regardless of the presence of a β -lactamase gene [14].

Embryogenic cultures

Embryogenic Norway spruce culture NS3 was initiated as described by Verhagen and Wann [44]. Cell

suspensions of early-staged embryos were grown in medium 471 (Table 2), a modification of BM3 [16] with 3% maltose substituted for sucrose. Cultures were grown on a seven day cycle in the dark at 20 °C on a rotary shaker at 100–110 rpm. Weekly transfers were accomplished by settling cells for 20 min, removing spent supernatant medium and mixing one part of settled cells with nine parts of liquid medium 471. To supply ample cells, cultures were most often grown in 1 liter flasks with 30 ml cells and 270 ml medium. Towards the end of this study a patent was issued on the use of maltose to maintain embryogenic conifer tissue [15]. Embryogenic loblolly pine culture 266 was maintained on medium 16 (Table 2, [32]) as described above.

Agrobacterium-mediated transformation

Transformation experiments were done 5 days after subculture. Ca. 20 ml of suspension-cultured cells were transferred to sterile 50 ml tubes. The volume was adjusted to 50 ml with 471 medium. *Agrobacterium*, grown for either 1 or 2 days (depending on the strain) to an optical density ($\lambda = 600$ nm) of ca. 1 in 3 ml of YEP [2] broth, were centrifuged and re-suspended in plant medium. One half of the cells from the original 3 ml cultures (ca. 10^8 – 10^9 bacteria) was added to the 50 ml tube. For standard transformations, acetosyringone (Sigma Chemical, St. Louis, MO) was added to a final concentration of 50 μ M. The cultures were then transferred to 250 ml Erlenmeyer flasks and allowed to settle for 1 h before being returned to the shaker. Co-cultivation was carried out for 2 days. *Agrobacterium* was removed from the cultures by allowing suspensions to settle in 50 ml sterile tubes and pouring off medium. The medium was replaced with fresh 471 medium containing 200 mg/l Timentin (ticarcillin/clavulanic acid 3:0.1, SmithKline Beecham, Philadelphia, PA). The wash was repeated twice. Ca. 4 ml of cells were transferred to plates containing solidified 471 medium (0.2% Phytigel, Sigma Chemical, St. Louis, MO) with 400 mg/l Timentin and 10 mg/l kanamycin sulfate (Sigma Chemical) and incubated in the dark at room temperature (ca. 25 °C). For GUS staining, 0.5 or 1 ml of cells were placed in 1 ml of GUS histochemical buffer [24] and incubated overnight at 37 °C. Transient expression was determined by counting the number of blue cells per ml of cultured cells 2 or 3 days after co-cultivation.

To select stable transformants, 3–4 ml of suspension cultures were directly plated on plates containing

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Table 1. *Agrobacterium* strains and plasmids utilized in this study.

Strain	Chromosomal background	Opine type	Reference
LBA 4404	Ach5	Octopine	[20]
GV 3101 (pMP 90)	C58	Nopaline	[27]
EHA 105	C58	Succinamopine	[21]
Plasmid	Origin of replication	Antibiotic selection	Description
pAD 1289	RK1	Carbenicillin (100 mg/l)	Constitutively active <i>virG</i> [18]
pToK 47	pSa	Carbenicillin (100 mg/l) Tetracycline (5 mg/l)	15.8 kb <i>KpnI</i> fragment from pTiBo542 containing <i>virB</i> , <i>virC</i> and <i>virG</i> [25]
pBISN1	RK2	Kanamycin (50 mg/l)	Binary vector with kanamycin selection (10 mg/l) and an intron containing GUS gene [31]
pWWS 006	PSV1	Spectinomycin (100 mg/l)	Binary vector with kanamycin (10 mg/l) and hygromycin (2.5 mg/l) selection (this work)

kanamycin (10 mg/l). After 3 weeks, putative transformants were transferred to fresh kanamycin-containing plates. After another 3 weeks, surviving embryogenic cells were again transferred to fresh plates. After a third round of selection, embryogenic cells were again transferred to fresh plates. At this time, a portion of each clump of cells was examined for homogeneity in the culture and the presence of potential 'escapes' in selected lines via GUS staining. Further subcultures were done biweekly on plates containing Timentin alone. Hygromycin selection (2.5 mg/l) was the same as for kanamycin selection except that the cultures were first plated on medium containing Timentin alone for 1–3 weeks prior to hygromycin selection.

To obtain large quantities of transformed tissues for further analysis, selected embryogenic cultures were again introduced into liquid suspension in medium 471 (Table 2). After 4–6 weeks, the cultures were actively producing 5–10 gm of tissue each week which was then used to prepare DNA for Southern blot analysis [32].

Transformation of loblolly pine was performed as described above for Norway spruce with the exception

that medium 16 (Table 2) was utilized in all of the transformation steps.

Embryo maturation

Mature embryos were obtained by plating selected cell lines on medium containing 471 macro-nutrients, micro-nutrients, vitamins and glutamine (Table 2), 6% sucrose, 0.4% Phytagel and either 15 or 30 μ M abscisic acid [43]. After 3 weeks the embryogenic masses were transferred to fresh ABA plates for another 3 weeks of maturation. Mature embryos were germinated on medium containing 1/2 DCR salts (Sigma Chemical) supplemented with 1% sucrose and solidified with 0.2% Phytagel.

DNA detection

Molecular analysis was performed on the selected cell lines using either a PCR approach on small samples or Southern analysis from large quantities of tissues. DNA was extracted from embryogenic cultures using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) following the manufacturer's protocol for DNA extraction from fresh plant tissues.

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Table 2. Composition of Norway spruce (471) and loblolly pine (16) media. Macronutrients (NH_4NO_3 - FeSO_4) along with the myo-inositol and casamino acids were added as a 20× stock. Micronutrients (KI - CoCl_2) were added as a 100× stock. Plant growth regulators: 2,4 dichlorophenoxyacetic acid (2,4-D), 6-henzylaminopurine (BA) and kinetin were added from 1000× stocks. Vitamins (100× stock: thiamine HCl - glycine) and glutamine (32 mg/l stock) were added after autoclaving.

Chemical	Medium 471*	Medium 16*
NH_4NO_3	206.5	603.8
KNO_3	2.340	909.9
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	185.0	246.5
KH_2PO_4	85.0	136.1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	85.0	0.0
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.0	236.2
$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	0.0	256.5
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.0	101.7
Na_2EDTA	18.65	9.33
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	13.9	6.95
KI	0.42	4.15
H_3BO_3	3.1	15.5
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	8.45	10.5
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	4.3	14.4
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.125	0.125
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.012	0.125
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.012	0.125
Maltose	30,000	0.0
Sucrose	0.0	30,000
Myo-inositol	1,000	1,000
Casamino acids	500	500
L-Glutamine	450	450
Thiamine HCl	1.0	1.0
Pyridoxine HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2.0	2.0
2,4-D	1.1	1.1
BA	0.45	0.45
Kinetin	0.43	0.43
pH	5.5-5.8	5.5-5.8

*Expressed as mg L⁻¹.

Large-scale preparations utilized 3–5 g (fresh weight) of cultured cells while small-scale preparations used 200–500 mg.

Standard PCR was carried out in 50 μl reactions containing ca. 100–500 ng of spruce DNA, 15 pmol of each primer, 200 μM each dNTP, 1× PCR buffer (1.5 mM MgCl_2 ; Boehringer Mannheim, Indianapo-

lis, IN) and 1 unit *Taq* DNA polymerase. Primer sets utilized were 5'-TCAAGCGCTGTGAACAAGG-3' and 5'-GATCAACAAACCACGACATCG-3' for *virG* of *Agrobacterium* Ti plasmid pTiBo542; 5'-TTCAGCTTCGATGTAGGAGG-3' and 5'-AGAAG-AAGATGTTGGCGACC-3' for the hygromycin phosphotransferase gene (HPT); and 5'-ACAACAGACA-ATCGGCTGC-3' and 5'-AAGAACTCGTCAAGAA-GGCG-3' for the neomycin phosphotransferase (NPT) gene. The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 52 °C for 1 min and 72 °C for 2 min. Cycling was followed with an ending step of 72 °C for 10 min followed by 4 °C using a MJ MiniCycler (MJ Research, Watertown, MA). PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining [34].

For Southern analysis, 15–30 μg of DNA was digested with 5 units of *Eco*RI for 6–8 hours at 37 °C and was subsequently separated on a 0.7% agarose gel. DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) in alkali [34]. DNA was bound to the membranes using a UV Stratalinker (Stratagene, La Jolla, CA). Membranes were prehybridized as specified by the manufacturer (Boehringer Mannheim) in a hybridization oven (Robbins Scientific, Sunnyvale, CA.) at 65 °C. The probe used in the hybridization reaction was the HPT gene isolated as an *Eco*RI fragment from pTRA151 [49] and was labeled by the random priming method [12]. Hybridization was for 2 days at 65 °C followed by 2 non-stringent and 2 stringent washes (2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS, respectively; [34]).

An anchored PCR method was used as described by Siebert *et al.* [39] to isolate right-border junction fragments. The sequences of the adaptor (ADP-48), 3' lower-strand oligo with amino group (ADP-8BL), and adaptor primers (AP1 and AP2) were: 5'-GTAATACGACTCACTATAAGGGCACCGCTGGTC-GACGGCCCGGGCTGGT-3' (ADP-48), 5'-ACCA-GCCC-N₂H-3' (ADP-8BL), 5'-CCATCCTAACAG-ACTCACTATAAGGGC-3' (AP1) 5'-CTATAGGGCA-CGCGTGGT-3' (AP2). The right-border T-DNA specific primers used were: 5'-AACCTGGCGTTACC-CAACTAAC-3' (PR8587) and 5'-CGCACCGAT-CGCCCTTCCCAACAGT-3' (PR8662).

To prepare the adaptor for ligation of digested DNA, ADP-8BL was phosphorylated using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). ADP-48 was then annealed to ADP-8BL at 50 °C for 1 min and allowed to cool slowly (1 °C per 90 s) down to 10 °C.

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The adaptor ligated DNA was prepared by digesting 2 µg of DNA from selected cell lines overnight at 37 °C in 150 µl reaction volume with 60 units of *Dra*I, *Eco*RI, or *Scal* (Promega, Madison, WI). Digestions were checked for completion by running 133 ng of each on a 0.8% agarose gel by standard techniques. The digests were then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. After centrifugation, pellets were washed with 70% ethanol, dried and resuspended in 18.6 µl sterile dH₂O. One µg of this DNA was then ligated to 100 ng of prepared adaptor at 8 °C overnight using T4 DNA ligase and buffers from the supplier (New England Biolabs), in a total reaction volume of 20 µl. The ligation reaction was terminated by incubating at 70 °C for 5 min, diluted 10-fold by adding 180 µl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA and stored at -20 °C until use.

Primary PCR reactions were set up in 50 µl reaction volumes containing 1 µl of ligated and diluted DNA, 20 mM Tris-HCl pH 9.0, 16 mM (NH₄)₂SO₄, 3.5 mM MgCl₂, 400 µM dNTPs, 0.5 µM each of adaptor primer (AP1) and right-border T-DNA specific primer (PR8587) and 2.5 units *Taq* DNA polymerase, (rTaq: Takara, Kyoto, Japan). The cycling was done in a programmable thermal controller equipped with a hot bonnet (MJ Research). The parameters used were: initial denaturation at 94 °C for 5 s, then a step down procedure was used with denaturation at 94 °C for 5 s, annealing at 72 °C for 3 min for 5 cycles stepping down 1 °C per cycle to 67 °C, then denaturation at 94 °C for 5 sec, annealing at 67 °C for 3 min for 40 cycles, then a final annealing/extension at 67 °C for 4 min.

A secondary PCR reaction was set up with 1 µl of a 100-fold dilution of the primary PCR reaction using adaptor primer, AP2 and the nested right border (T-DNA-specific) primer, PR8662. The same reaction and cycle parameters were used except 20 thermocycles were performed after the initial 5 cycles stepping down at 67 °C.

All PCR products were separated on 0.8% agarose gels by standard techniques. PCR products were either sequenced directly using the PR8662 and AP2 primers or by first cloning them into the vector pT7Blue-2 (Novagen, Madison, WI) and then sequencing with universal primers. Sequencing of PCR products was done at the Iowa State sequencing facility. Cloned products were sequenced at the North Carolina State University sequencing facility using a Li-Cor system.

Results

Effects of Agrobacterium strains and extra virulence genes on Norway spruce transformation

Transient expression, determined as the number of GUS positive cells/mL of embryos two or three days following co-cultivation, was significantly increased when additional copies of the *virG* and *virB* regions from pTiBo542 (pToK47) were added to *Agrobacterium* (Table 3). The increase was strain specific with no change in GV3101 but over a 400-fold increase with LBA 4404 and a 10-fold increase with EHA 105. Transient expression was not increased with additional copies of a constitutively active *virG* gene (pAD1289). Selection efficiencies (determined as the percent of transient expressing cells plated that gave rise to transformed embryogenic cultures after 3 rounds of selection (9 weeks) ranged from 0.01-0.08% and were 2-3-fold higher for the experiments in which the agrobacteria contained either extra copies of virulence genes (pToK47) or constitutive expression of virulence (pAD1289; data not shown).

Putative transformants appeared within 3 weeks of plating onto kanamycin containing plates. After 2 more rounds of selection, portions of each colony were screened for GUS expression and 94% were found to express GUS throughout most or all of the cells. GUS-like staining was never observed in untransformed cultures or in cultures transformed with a binary vector that did not contain the GUS gene. Stable transformed lines were induced to form mature embryos for germination. GUS expression was not detectable during maturation on ABA-containing medium; however, expression returned upon germination of the embryos. The period of kanamycin selection described above did not inhibit embryo maturation as previously described for longer periods of selection [33]. Hygromycin selection (2.5 mg/l) was also used with certain transformations, but a period of at least 1 week was required after co-cultivation before selection could be applied. Efficiencies were the same for kanamycin and hygromycin selection.

Effect of acetosyringone and bacteria concentration on Norway spruce transformation

Transformation was influenced by the presence of the inducer acetosyringone, with 25-50 µM being optimal (Table 4). A slight phytotoxic effect was observed with the 100 µM treatment and may contribute to the

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Table 3. Transformation efficiencies of different *Agrobacterium* strains as determined by the number of GUS-positive spots 2 or 3 days after co-cultivation.

Strain	Norway spruce*	Loblolly pine*
EHA 105 (pBISN1)	152 (88)	11 (5)
EHA 105 (pBISN1, pAD1289)	110 (24)	nd**
EHA 105 (pBISN1, pToK47)	1224 (199)	121 (34)
GV 3101 (pBISN1)	470 (108)	21 (21)
GV 3101 (pBISN1, pAD1289)	488 (70)	nd
GV 3101 (pBISN1, pToK47)	410 (122)	6 (1)
LBA 4404 (pBISN1)	0	nd
LBA 4404 (pBISN1, pToK47)	459 (69)	29 (15)

* Mean number of GUS positive spots per ml of cells. The total number of replications for each experiment was 5, 4, 4, 8, 4, 2, 4 and 12, respectively, for Norway spruce and 2, 5, 2, 4 and 4, respectively, for loblolly pine. Numbers parenthesis are standard errors.

** Not determined.

decrease in transformation observed at this higher concentration. Preinduction of the *Agrobacterium* with acetosyringone (200 µM) in plant medium (pH 5.6, sucrose or maltose 30 gm/l) at 20 °C did not lead to an increase in transient expression.

The amount of *Agrobacterium* used in the co-cultivation and the time of co-cultivation were both important factors. Less than 10⁸ bacteria was ineffective at transformation. Higher levels were not tested. Co-cultivation of less than 2 days resulted in no transient expression. Although longer co-cultivation times and higher bacteria concentrations were not tested, a 2-day co-cultivation was considered the maximum for these cells in that excessive bacterial growth resulted in death of the culture.

Molecular analysis of transformed Norway Spruce lines

PCR was used to examine some of the selected lines for the presence of the T-DNA. Of the lines selected, all analyzed showed the presence of T-DNA using the NPT or the HPT primer pairs (data using the NPT primer pair is shown in Figure 1). In addition, the *virG* primers were used to check for the presence of contaminating *Agrobacterium* that would have also given the same T-region band. In 2 of the 5 samples shown in Figure 1 a faint *virG* band was observed; however, the intensity of this band in comparison with the NPT band is greatly reduced compared to the band intensities when the 2 genes are present in an approximate 1:1 ratio (Figure 1, lane 8). These faint bands are proba-

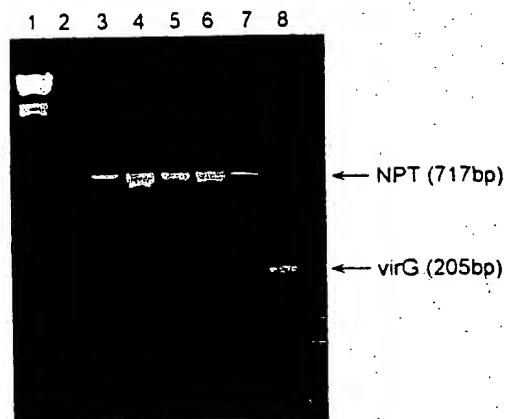


Figure 1. PCR detection of T-DNA sequences. About 100 ng of spruce DNA was subjected to PCR with the NPT and *VirG* primer sets as described in Materials and methods, separated on a 1.5% agarose gel and visualized with ethidium bromide staining. Lane 1: λ-DNA HindIII digest; lane 2: untransformed Norway spruce DNA; lanes 3–7: DNA from Norway spruce lines transformed with *Agrobacterium* EHA105 (pToK47, pBISN1); lane 8: pToK47 and pBISN1 plasmid DNA (ca. 10 pg each).

bly from a trace level of *Agrobacterium* contamination remaining in these cultures.

Southern analysis was performed on several of the selected lines (Figure 2). Single-copy insertion events can be identified by the presence of a single band of variable size when *Eco*RI, which cuts once within the T-DNA, and the HPT probe, which is at the right border, are utilized. Single copy insertion events were detected in 2 of 6 lines analyzed (5 lines are shown in figure 2). The other lines showed multiple-copy insertions. Two of the lines which showed multiple

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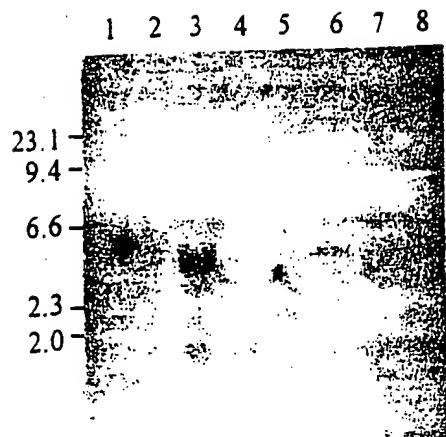


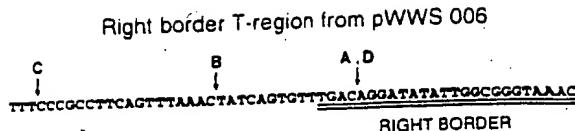
Figure 2. Southern detection of T-DNA sequences. Norway spruce lines were transformed with *Agrobacterium* containing plasmid pWWS006 which contains both the NPT and HPT genes. 15 µg of spruce DNA was digested with EcoRI, separated using an agarose gel and transferred onto a nylon membrane as described in Materials and methods. The probe used was the HPT gene labeled and hybridized as described in Materials and methods. Lane 1, λ -HindIII-digested DNA; lane 2: untransformed spruce DNA; lane 3, transformed line 006-1; lane 4, transformed line 006-2; lane 5, transformed line 006-3; lane 6, transformed line 006-4; lane 7, transformed line 006-6; lane 8, binary plasmid pWWS 006 (5 pg).

insertions had bands consistent with the presence of at least two T-DNAs in a head to tail configuration (Figure 2, lane 3 and not shown). This configuration was confirmed by anchored PCR analysis (see below).

Anchored PCR was utilized to isolate right border insertion fragments from 4 of the transformed lines. Two of these lines have plant/T-DNA junction fragments (Figure 3). The other two lines had T-DNA/T-DNA junction fragments consistent with the Southern data above.

Loblolly pine

Transient expression in loblolly pine was greatly influenced by the *Agrobacterium* strain utilized and by the presence of acetosyringone (Tables 3 and 4). Extra copies of the *virB* and *virG* regions of pTiBo542 increase transformation 10-fold for EHA 105. This was the only strain that had high levels of transient expression (Table 3). Extra copies of the constitutively expressed *virG* were not tested. Acetosyringone was required for high transformation efficiencies with 50 µM being optimal but not significantly different from 25 or 100 µM (Table 4). We have not been able to recover stable transformants through selection to date; however, GUS expression is observed when cultures are first kept on timentin containing medium for 4



- A. TTAACATATCAGTGTGACGCTCAGACAACTTAAACA
- B. TTCCCCCTTCAGTTAAACGAAACATTGTTAAATTGACA
- C. TTGACAGTATAATTGGCGGTAAAC
- D. TTAACATATCAGTGTGACGCTTAGACAGCTTAACA

Figure 3. Right-border junctions isolated using anchored PCR as described in Materials and methods. The right-border region from pWWS 006 including the right border (double underline) and break sites in the T-DNA are shown. Forty bp of sequence from each anchored PCR fragment, including 20 bp from the T-DNA (underlined) are given for lines 006-1, 2, 4 and 5. A. line 006-1; B. line 006-2; C. line 006-4; D. 006-5 (head-to-tail T-DNA insertion: see text).

Table 4. The influence of acetosyringone on *Agrobacterium*-mediated gene transfer.

Acetosyringone concn. (µM)	Norway spruce*	Loblolly pine*
0	58 (58)	0 (0)
25	574 (90)	41 (4)
50	512 (38)	97 (60)
100	276 (152)	95 (31)

*Mean number of GUS-positive spots per ml of cells for *Agrobacterium* LBA 4404 (pBISN1, pToK47; Norway spruce) or EHA 105 (pBISN1, pToK47; loblolly pine). Each acetosyringone concentration was replicated twice. Numbers in parenthesis are standard errors.

weeks after co-cultivation. In this case, the amount of expressing cells is only 33% of what is found when staining is done 2 days after co-cultivation.

Discussion

Several factors are important for *Agrobacterium*-mediated transformation of plants. These include source material, bacterial strain and co-cultivation conditions. The source material utilized here is rapidly dividing embryogenic suspension cultures. Embryogenic cultures were also utilized for *Agrobacterium*-mediated transformation of Sitka spruce [9] and white pine (*Pinus strobus*; [28]). Rapid growth of the source material is important for transformation of tobacco (*Nicotiana tabacum*; [1]), *Arabidopsis* [35] pine (*Pinus radiata*; [4]) and *Petunia* [45] and may contribute to the success observed in this study. Bergmann

and Stomp [4] found that rapid cell division was an even more important factor than genotype for *Agrobacterium*-mediated transformation of *P. radiata*. The exact timing of co-cultivation has not been thoroughly studied but 5–6 days after subculture roughly corresponds to the best time for biolistic transformation [48]. Co-cultivation in liquid may also have been beneficial. Although these embryogenic cultures can be grown and maintained on solidified medium, these cultures grow much better in liquid medium. Attempts at selection in liquid were unsuccessful, so solidified medium was always used for selection.

Acetosyringone also was essential for high transformation efficiency; however, the high levels used in some other systems (100 µM or greater) were not beneficial (Table 4). A phytotoxic effect was observed with higher levels (with Norway spruce only) but this may be due to the solvent used (ethyleneglycol monoethyl ether) instead of the acetosyringone itself.

The amount of *Agrobacterium* and the co-cultivation time were both important factors and have been shown to be important in many other transformation systems [7, 30] including sitka spruce [9]. The bacterial concentration used here corresponds well to the optimum found in other systems [30].

Strain selection is clearly important as has been shown for other species [reviewed by 14, 21]. Interestingly, the best unmodified disarmed helper strain for Norway spruce transformation is a disarmed nopaline strain (C58; Table 3). This strain was also recently used successfully for transformation of white pine embryogenic cultures [28] and wheat [5]. The wild-type C58, however, was not shown to be advantageous in gall formation on conifer stems in previous studies [11, 38, 40].

The addition of a constitutively active *virG* gene did not increase transient expression in the strains tested; however, additional copies of the *virG* and *virB* genes from the plasmid pTiBo542 (pToK 47) greatly increased transient expression of both the agropine (EHA 105) and the octopine (LBA 4404) strains. No effect was noticed when the nopaline (GV 3101) strain was used (Table 3). Jin *et al.* [25] observed that extra copies of *virG* increased transformation when placed into 'non-super-virulent' strains, but that *virB* was also required to increase transformation of the super-virulent strain A281. The requirement for extra copies of *virB*, which has been implicated in T-DNA transfer [13 and reviewed by 50], suggests that DNA transfer and not just virulence induction may be a limiting factor in transformation.

Previous results using similar constructs have shown that different combinations of this region and helper strains are better with some species than with others [30]. Clearly EHA 105 (pToK47) is the most efficient strain for transformation of both loblolly pine and Norway spruce embryogenic cultures. However, a similar combination of strains and plasmids was not superior when used for transformation of spruce stems [11]. With Sitka spruce, transient expression was observed with LBA 4404 (pToK47), but data for LBA 4404 was not shown nor were other combinations of strains and virulence factors analyzed [9].

The pSa origin of replication of pToK47 allows it to co-exist within the same *Agrobacterium* as 'standard' RK2 origin of replication binary vectors since they are in different incompatibility groups (IncW and IncP respectively; reviewed by [3]). Komari [26], however, was unable to obtain *Agrobacterium* containing these two plasmids and, therefore, created the 'super-binary' vectors utilized in that and other reports [19, 23, 29]. These super binary vectors contain the *VirB* and *virG* genes from pTiBo542 on the same plasmid as the T-region which makes it very large. Changes to the super binary are made in *Agrobacterium* by homologous recombination in a method reminiscent of the co-integrate vector systems of the past [reviewed by 47]. We and others [6, 9] have obtained *Agrobacterium* with both the pToK47 and binary plasmids as separate replicating plasmids. *Agrobacterium* containing both replicons are growing more slowly than 'standard' strains and are much slower to appear on transformation plates (Wenck, unpublished results). The overall transformation efficiency of the *Agrobacterium* is also lower. These factors may account for the inability of Komari [26] to obtain *Agrobacterium* containing both replicons.

The number of T-DNA insertions and the arrangement of insertions (head to tail) is in good agreement for *Agrobacterium*-mediated transformation [8]. Although only 6 lines were analyzed by Southern blotting, 2 of these had single-copy insertions (Figure 2 and not shown). Sequencing of the right-border junction in four of the lines indicated a slight truncation from the processing site of the right border in two of them (Figure 3B and C). Processing of the left border is revealed in the two head-to-tail insertions isolated (Figure 3A and D). Both of these are truncated within 8 bp of the left border sequence. Even though the isolated fragment from these head to tail insertions have the same right border and left border processing

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points, sequencing and Southern analysis show them to be independent lines.

With careful selection of the culture conditions (the use of rapidly dividing embryogenic suspension cultures) and the strains, we have increased transformation from an average of 0 ml (LBA 4404) to over 1000/ml (EHA 105 (pToK 47)) in Norway spruce and from 6/ml (GV 3101 (pToK 47)) to over 100/ml (EHA 105 (pToK 47)) in loblolly pine. The use of strains and techniques described here may lead to efficient transformation of other conifer species in the future.

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